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Engineered Tumor-Targeted T Cells Mediate Enhanced Anti-Tumor Efficacy Both Directly and through Activation of the Endogenous Immune System

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SUMMARY

Chimeric antigen receptor (CAR) T cell therapy has proven clinically beneficial against B cell acute lymphoblastic leukemia and non-Hodgkin's lymphoma. However, suboptimal clinical outcomes have been associated with decreased expansion and persistence of adoptively transferred CAR T cells, antigen-negative relapses, and impairment by an immunosuppressive tumor microenvironment. Improvements in CAR T cell design are required to enhance clinical efficacy, as well as broaden the applicability of this technology. Here, we demonstrate that interleukin-18 (IL-18)-secreting CAR T cells exhibit enhanced *in vivo* expansion and persistence and significantly increase long-term survival in syngeneic mouse models of both hematological and solid malignancies. In addition, we demonstrate that IL-18-secreting CAR T cells are capable of modulating the tumor microenvironment, as well as enhancing an effective endogenous anti-tumor immune response. IL-18-secreting CART cells represent a promising strategy to enhance the clinical outcomes of adoptive T cell therapy.

Graphical abstract

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at https://doi.org/10.1016Zj.celrep.2018.04.051.

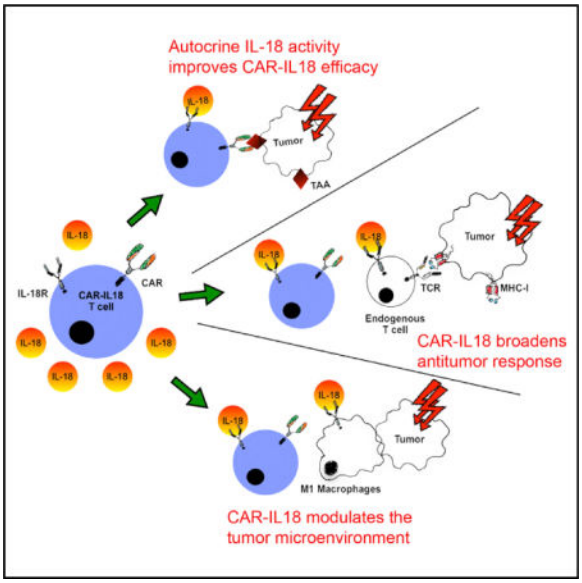
AUTHOR CONTRIBUTIONS

M.P.A. and R.J.B. conceived the study. M.P.A., O.Y., and R.J.B. designed the study and analyzed the data. M.P.A., O.Y., and X.L. performed most ofthe experiments, with contributions from D.P.W., D.G.v.L., K.C., H.P., T.J.P., and A.F.D. M.H.S. supervised and analyzed the mass cytometry data. M.P.A., O.Y., and R.J.B. wrote the manuscript, with contributions from A.F.D. and M.H.S.

DECLARATION OF INTERESTS

R.J.B. is a co-founder and receives royalties from Juno Therapeutics. R.J.B. and M.P.A. have a pending patent application based on this work.

In Brief Avanzi et al. generate CAR T cells that secrete IL-18 and show improved activity in syngeneic hematologic and solid tumor models without prior preconditioning. They further show enhanced recruitment and anti-tumor activity of endogenous T cells.



INTRODUCTION

Adoptive T cell therapy with chimeric antigen receptor (CAR) T cells has emerged as an effective therapy for the treatment of B cell hematological malignancies, and several groups have published results utilizing anti-CD19 CAR T cells for the treatment of B cell acute lymphoblastic leukemia (B-ALL), and non-Hodgkin's lymphoma (NHL) (Brentjens et al., 2013; Davila et al., 2014; Lee et al., 2015; Maude et al., 2014). However, despite high rates of initial complete remissions, a considerable number of patients will relapse with either $CD19^{+}$ or $CD19^{-}$ disease after CD19-targeted CAR T cell therapy (Gardner et al., 2016; Maude et al., 2014; O'Rourke et al., 2017). Relapses that retain surface CD19 expression are thought to result from decreased persistence and/or decreased function of CAR-modified T cells. Unsurprisingly, increased circulating CAR T cell persistence correlates with durable responses and enhanced clinical outcomes (Kalos et al., 2011; Maude et al., 2014). Relapses may also occur secondary to emergence of tumor cells that have lost CD19 expression, despite persistence of functional CAR T cells. The incidence of relapses with antigen loss is related to escape variants (Sotillo et al., 2015), and according to recent estimates, epitope loss accounts for up to 40% of reported relapses (Gardner et al., 2017; Maude et al., 2014; O'Rourke et al., 2017). In addition, CAR T cells have demonstrated limited efficacy for the treatment of other hematological malignancies, such as chronic lymphocytic leukemia (CLL), as well as solid tumor malignancies (Brown et al., 2016; Feng et al., 2017; Jackson et al., 2016; Louis et al., 2011; O'Rourke et al., 2017; Wang et al., 2015).

Emerging evidence suggests that an immunosuppressive tumor microenvironment may lead to early dysfunction, decreased expansion, and poor persistence of adoptively transferred T cells (Cherkassky et al., 2016; Gajewski et al., 2006; John et al., 2013). CAR T cells capable

of overcoming these limitations are needed in order to improve clinical outcomes, decrease relapses, and expand the spectrum of diseases treated with this technology.

Interleukin-18 (IL-18) is an IL-1 family cytokine produced by macrophages that directly stimulates interferon-γ (IFN-γ) secretion, and has pleiotropic effects on cells of the endogenous immune system. This property makes IL-18 a promising candidate for enhancing the anti-tumor efficacy of genetically modified T cells. In fact, IL-18-secreting CAR T cells have recently been shown to improve anti-tumor efficacy in a xenogeneic mouse model of $CD19^+$ hematologic malignancies (Hu et al., 2017). However, due to the lack of an intact host immune system in these mice, the efficacy of this approach in the presence of an immunosuppressive tumor microenvironment remains unknown. In a more recent study, IL-18-secreting CAR T cells eradicated established pancreatic cancer and metastatic lung cancer in syngeneic and xenogeneic pre-clinical solid tumor models, respectively (Chmielewski and Abken, 2017).

In this study, we demonstrate that CAR T cells engineered to secrete IL-18 exhibit enhanced proliferation and persistence, and significantly increase long-term survival in syngeneic mouse models of both hematologic and metastatic solid tumor malignancies. We further demonstrate that this effect is largely dependent on autocrine IL-18 signaling. Finally, we show that IL-18 armored CAR T cells are capable of recruiting an effective and comprehensive endogenous anti-tumor immune response.

RESULTS

Human IL-18-Secreting CAR T Cells Display Enhanced Proliferation and Prolong Survival in a Xenograft Scid-Beige Mouse Model

We generated the human CD19-targeted 1928z-hIL18 CAR retroviral construct from a previously described and clinically utilized 1928z CAR construct (Brentjens et al., 2003). Ovarian tumor-targeted anti-$Muc16^{ecto}$ 4H1128z CAR T cells were utilized as untargeted controls (Figure 1A) (Chekmasova et al., 2010). The 1928z-hIL18 CAR showed comparable gene transfer to 1928z CARs (Figure S1A). Both 1928z-hIL18 and 1928z CAR T cells had similar $CD4^+$ and $CD8^+$ populations, with the majority of the transduced cells being $CD8^+$ (Figure S1B). There were no differences in central/effector memory phenotypes between 1928z-hIL18 and 1928z CAR T cells (Figure S1C). To validate the construct, human T cells modified to express the 1928z-hIL18 CAR vector were compared to 1928z CAR T cells and demonstrated enhanced *in vitro* IL-18 ($p = 0.004$), IFN-γ ($p < 0.0001$), and IL-2 ($p = 0.0003$) secretion after stimulation with $CD19^+$ NALM6 B-ALL tumor cells (Figure 1B). In addition, 1928z-hIL18 CAR T cells compared to 1928z CAR T cells demonstrated enhanced proliferation (Figure 1C) ($p = 0.003$) and retained anti-tumor cytotoxicity (Figures 1D and 1E) after NALM6 tumor stimulation. No difference in markers associated with T cell dysfunction were observed between 1928z and 1928z-hIL18 CAR T cells *in vitro* on days 7 and 14 (Figure S1G). Next, we examined the gene expression profile of both CAR T cells at day 1 and day 8 after co-culture with tumor cells using the nanoString PanCancer Immune Profiling Panel. Only genes with statistical significance are shown ($p < 0.05$). Overall, compared to 1928z, 1928z-hIL18 T cells exhibited increased expression of pro-inflammatory and activation genes (IFN-γ and PD1) and decreased expression of pro-

apoptotic genes (Pycard and ETS1) on day 1 after tumor stimulation (Figure 1F). After 8 days of coculture, pro-inflammatory genes (MAPK8 and TNFαIP3) and genes indicative of a central memory phenotype (CCR7) were selectively upregulated in 1928z-hIL18 CAR T cells (Figure 1G), compared to 1928z CAR T cells. In order to assess the *in vivo* anti-tumor efficacy of 1928z-hIL18 CAR T cells, we treated NALM6-GFP$^+$/Luc$^+$ tumor-bearing Scid-Beige mice with 1928z or 1928z-hIL18 CAR T cells. This tumor model has been previously demonstrated to be extremely aggressive and multiple second-generation 1928z CAR T cell injections were necessary to obtain long-term anti-tumor efficacy (Brentjens et al., 2007). Mice were inoculated with 1×10^6 NALM6-GFP$^+$/Luc$^+$ tumor cells on day 0 and treated with 5×10^6 CAR T cells on day 1. As demonstrated, 1928z-hIL18 CAR T cells significantly enhanced survival compared to 1928z ($p = 0.0006$) (Figure 1H) and significantly lowered tumor burden as assessed by bioluminescent imaging (region of interest [ROI]) (Figure 1I).

Murine IL-18-Secreting CAR T Cells Enhance Survival of EL4hCD19$^+$ Tumor-Bearing Syngeneic Immunocompetent Mice in the Absence of Chemotherapy Preconditioning

Given that immunocompromised xenotransplant mouse tumor models have limited clinical and biological relevance, we next studied IL-18-secreting CAR T cells in the context of syngeneic, immune competent models of disease wherein a more comprehensive analysis of the anti-tumor effects mediated by murine IL-18 (mIL-18)-secreting T cells could be assessed. To this end, we generated a panel of retroviral vectors encoding murine CAR T cells that target hCD19: 19m28mz-mIL18, 19mz-mIL18, 19mDel-mIL18, and 19mDel all derived from a 19m28mz retroviral construct (Figure 2A). Murine 19m28mz-mIL18 showed comparable gene transfer to 19m28mz (Figure S1D). There were no differences in CD4/CD8 ratios (Figure S1E) or effector/central memory phenotypes (Figure S1F) between both groups. As predicted, mouse T cells modified with the 19m28mz-mIL18 retroviral vector demonstrated enhanced *in vitro* IL-18 secretion ($p = 0.005$) when cocultured with a thymoma tumor cell line (EL4), modified to express hCD19 (EL4hCD19$^+$) (Figure 2B). 19m28mz-mIL18 CAR T cells showed comparable expansion and anti-tumor cytotoxicity compared to 19m28mz CAR T cells (Figures S2A and S2B). Utilizing a syngeneic hCD19$^+$ transgenic mouse model (C57BL/6 mCD19$^{+/-}$ hCD19$^{+/-}$), we evaluated the efficacy of 19m28mz-mIL18 CAR T cells in mice infused systemically with EL4hCD19$^+$ tumor cells. We have previously shown that EL4hCD19$^+$ tumor-bearing mice treated with anti-hCD19 CAR T cells in the absence of prior cyclophosphamide preconditioning do not demonstrate tumor eradication and long-term survival (Pegram et al., 2012). In these experiments, CAR T cells failed to eradicate tumor cells and all mice succumbed to disease at early time points (Pegram et al., 2012). We used this aggressive tumor model to investigate whether 19m28mz-mIL18 CAR T cells could overcome the lack of anti-tumor efficacy exhibited by second-generation 19m28mz T cells. In the absence of preconditioning, 19m28mz-mIL18 CAR T cells (2.5×10^6 CAR cells/mouse) were capable of significantly enhancing long-term survival of EL4hCD19$^+$ tumor-bearing syngeneic mice, compared to 19m28mz CAR T cell treatment ($p < 0.0001$) (Figure 2C). Anti-tumor efficacy was readily evident even in the context of treatment with reduced 19m28mz-mIL18 CAR T cell dose (1.2×10^6 CAR cells/mouse) (Figure 2D). Furthermore, 19m28mz-mIL18 CAR T cells were capable of enhancing long-term survival in a delayed tumor model wherein CAR T cells were administered on day

7 after tumor inoculation ($p = 0.0009$) (Figure 2E). We next assessed whether 19m28mz-mIL18 CAR T cells persist and retain meaningful anti-tumor efficacy against tumor rechallenge. Surviving mice previously inoculated with 1×10^6 EL4hCD19$^+$ tumor cells on day 0 and treated with 19m28mz-mIL18 CART cells on day 1 were re-challenged with a second injection of 1×10^6 tumor cells 40 days after the first tumor injection. Mice treated with 19m28mz-mIL18 CAR T cells were capable of rejecting a second lethal dose of tumor ($p = 0.004$) (Figure 2F). Significantly, mice that succumbed to disease in these studies harbored CD19$^+$ tumor cells on necropsy.

Murine IL-18-Secreting CAR T Cells Exhibit Enhanced Expansion and Persistence and Induce Prolonged B Cell Aplasia Dependent on Autocrine IL-18R Signaling

19m28mz-mIL18 CART cells displayed enhanced *in vivo* expansion and were detected in peripheral blood by flow cytometry for up to 28 days after infusion, whereas 19m28mz CAR T cells were not detected in peripheral blood at any time point tested (Figure 3A). *In vivo*, 19m28mz-mIL18 CAR T cells were predominantly CD8$^+$ (Figure S3A), similar to increased CD8/CD4 ratios pre-infusion. We have previously demonstrated that hCD19$^+$ transgenic mice develop B cell aplasia after treatment with CD19-directed CAR T cells (Pegram et al., 2012). B cell aplasia in this mouse model is an effective surrogate marker for CD19 targeted CAR T cell activity and directly correlates with the presence of CAR T cells either in circulation or in the bone marrow (Pegram et al., 2012). Mice treated with 19m28mz-mIL18 CAR T cells developed relative and persistent B cell aplasia for up to 150 days after treatment, whereas mice treated with 19m28mz CAR T cells did not develop B cell aplasia at any time point (Figure 3B). Untreated tumor-bearing mice had similar B cells compared to 19m28mz- treated mice (Figure S3B), likely due to the inability of 19m28mz T cells to expand *in vivo* without lymphodepleting therapy. These data further serve to highlight the potency of IL-18-modified CARs in a syngeneic model without preconditioning. To further verify the persistence of 19m28mz-mIL18 CAR T cells over time and to validate the role of these CAR T cells in observed prolonged B cell aplasia, bone marrow aspirates were collected from 19m28mz-mIL18- treated mice and assayed for the CAR construct by PCR. 19m28mz-mIL18 CAR T cells were detected by PCR in the bone marrow at 35, 80, 120, and 150 days after infusion (Figure 3C), suggesting that the prolonged B cell aplasia was directly related to the enhanced persistence of CAR T cells in the bone marrow. In addition, we found that 19m28mz-mIL18 CAR T cells were capable of significantly enhancing serum levels of IL-18, IFN-γ, and tumor necrosis factor α (TNFα) at day 7, compared to 19m28mz CAR T cells ($p = 0.0002$, $p < 0.0001$, $p = 0.005$, respectively) (Figure 3D). These data provide a mechanistic basis for the rejection of a second round of tumor inoculation. Significantly, no increase in serum IL-6, a cytokine associated with cytokine release syndrome (CRS) in the clinical setting, was detected in mice treated with 19m28mz-mIL18 CAR T cells (Figure 3D).

We initially utilized a second-generation CAR T cell containing a CD28 signaling domain in our experiments. To evaluate if “signal 2” (CD28 costimulation) was dispensable for IL-18 armored CAR T cell anti-tumor efficacy and persistence, we engineered first-generation CAR T cells that lack the CD28 costimulatory signaling domain (19mz-mIL18) and directly compared these 19mz-mIL18 (signal 1+3) CAR T cells *in vivo* to second-generation

19m28mz-mIL18 CAR T cells (signal 1 + 2 + 3). First-generation 19mz-mIL18 CAR T cells were capable of inducing long-term survival; however, the overall survival benefit was inferior to that seen in mice treated with the second-generation 19m28mz-mIL18 CAR T cells (Figure 3E). In addition, 19mz-mIL18 CAR T cells demonstrated only modest expansion and induced only transient B cell aplasia in peripheral blood compared to mice treated with 19m28mz-mIL18 CAR T cells (Figures 3F and 3G).

Next, in order to determine whether the effects of 19m28mz-mIL18 CAR T cells were dependent on IL-18 secretion alone (signal 3), CAR-mediated T cell signaling (signal 1), or both concomitantly, we engineered constructs with deletion of the intracellular CD28 and CD3z signaling domains (19mDEL and 19mDEL-mIL18 CAR T cells). These T cells are capable of recognizing the tumor but do not display any signaling through the CAR and therefore no direct killing of the targeted tumor cells. Both 19mDEL and 19mDEL-mIL18 CAR T cells failed to enhance survival in EL4hCD19^{+} tumor-bearing syngeneic mice (Figure S4A). Taken together, in this model, signals 1, 2, and 3 are required for optimal anti-tumor efficacy. In order to determine whether the enhanced effect of IL-18-secreting CAR T cells was dependent on autocrine IL-18 stimulation, we compared the *in vivo* efficacy of 19m28mz-mIL18–transduced CAR T cells derived from IL-18R knockout mice splenocytes (C57BL/6 IL-18R$^{-/-}$) to 19m28mz-mIL18 CAR T cells derived from transgenic mice splenocytes (C57BL/6 mCD19$^{+/-}$ hCD19$^{+/-}$). CAR T cells derived from IL-18R$^{-/-}$ splenocytes do not have any deficits in cytokine production or cytotoxicity at baseline (Figures S4B and S4C). Mice treated with 19m28mz- mIL18 CAR T cells derived from IL-18R knockout splenocytes failed to enhance long-term survival, expansion, persistence, or B cell aplasia ($p = 0.001$; $p = 0.0003$; $p < 0.0001$, respectively) (Figures 3H, 3I, and 3J). Furthermore, serum IFN-γ levels were significantly lower in mice treated with IL18R$^{-/-}$ 19m28mz-mIL18 CAR T cells ($p < 0.0001$) (Figure 3K).

To confirm that our findings were not restricted to CD19^{+} hematological malignancies, we expanded our studies to a metastatic ovarian cancer solid tumor model. We generated murine retroviral constructs of our previously published 4H11-targeting CAR to the truncated MUC16 antigen (MUC16^{ecto}) (Chekmasova et al., 2010; Koneru et al., 2015) and conducted *in vivo* experiments. C57BL/6 syngeneic mice were inoculated with ID8 (MUC16^{ecto}) ovarian tumor cells and treated with 4H11m28mz or 4H11m28mz-mIL18 CAR T cells. Our results demonstrated that 4H11m28mz-mIL18 CAR T cells were capable of significantly enhancing long-term survival in both low and high tumor burden models of syngeneic metastatic ovarian carcinoma (Figures S5A and S5B). These studies exemplify the potential application of this IL-18-secreting adoptive CAR T cell approach to both hematologic and solid tumor malignancies.

IL-18-Secreting CAR T Cells Modulate the Tumor Microenvironment

To assess the capacity of 19m28mz-mIL18 CAR T cells to migrate to the tumor site as well as their effects on endogenous immune cells, we performed experiments in immunocompetent syngeneic mice and utilized mass cytometry (cytometry by time of flight [CyTOF]) to analyze bone marrow samples. EL4 tumor cells have been shown to migrate to and proliferate in the bone marrow (Krevvata et al., 2014; Richards et al., 2006). CyTOF

results demonstrated that, unlike 19m28mz CAR T cells, 19m28mz-mIL18 CAR T cells were capable of migrating to the bone marrow and were detectable at day 18 after intravenous (i.v.) injection (Figure 4A). Bone marrow analysis demonstrated that 19m28mz-mIL18 CAR T cells were indeed capable of not only significantly decreasing the B cell population (Figure 4B) but also of inducing expansion of bone marrow endogenous immune effector cells, such as natural killer (NK) cells, NKT cells, dendritic cells (DCs), and endogenous CD8 T cells, compared to 19m28mz CAR T cell-treated mice ($p = 0.03$; $p = 0.03$; $p = 0.03$; $p = 0.03$; $p = 0.03$, respectively) (Figure 4B). The increased CD8 T cell population in the bone marrow was mostly composed of endogenous CD8 non-CAR T cells (Figure 4C). More interestingly, 19m28mz-mIL18 CAR T cells were capable of modulating and activating endogenous immune cells residing in the bone marrow. Mice treated with 19m28mz-mIL18 CAR T cells, compared to mice treated with 19m28mz CAR T cells, displayed enhanced numbers of endogenous CD8 T cells with a central memory phenotype ($CD44^+$; $Ly6C^+$) ($p = 0.01$), macrophages with an M1 phenotype ($MHC\text{-}II^+$) ($p < 0.0001$), and DCs with a more mature and activated phenotype ($CD86^+$; $MHC\text{-}II^+$) ($p = 0.02$) (Figures 4D–4F). Also, while many endogenous and CAR^+ CD8 T cells exhibited characteristics of central memory cells ($CD44^+$; $Ly6C^+$), CAR T cells expressed higher levels of CD27, PD-1, and CD3 (Figure 4G).

IL-18-Secreting CAR T Cells Recruit Endogenous Anti-Tumor Immune Effector Cells and Broaden the Anti-Tumor Response Beyond the CAR Target

We postulated that 19m28mz-mIL18 CART cells could possibly be stimulating endogenous CD8T cells toward a central memory phenotype and thus enhancing the anti-tumor effect through recruitment of endogenous CAR^- tumor targeted T cells. To evaluate this hypothesis, we inoculated syngeneic mice with a mixed population of equal amounts of $EL4hCD19^+$ and $EL4hCD19^-$ tumor cells, followed by treatment with anti-CD19 19m28mz-mIL18 CAR T cells. Interestingly, 19m28mz-mIL18 CAR T cells were capable of enhancing long-term survival of mice inoculated with both $CD19^+$ and $CD19^-$ tumor cells at a 1:1 ratio (Figure 5A). This is consistent with recruitment of endogenous CD8 T cells targeted to antigens other than CD19 expressed by $CD19^-$ tumor cells. To further verify that 19m28mz-mIL18 CAR T cells recruit endogenous tumor targeted T cells, we conducted additional enzyme-linked immunospot (ELISPOT) experiments with fluorescence-activated cell sorting (FACS)-sorted $CD3^+$ CAR^- splenocytes (Figure S6A) derived from $EL4hCD19^+$ tumor-bearing mice treated with either 19m28mz or 19m28mz-mIL18 CART cells. ELISPOT results demonstrated that isolated CAR^- splenocytes derived from mice treated with 19m28mz-mIL18 CAR T cells exhibited enhanced levels of IFN-γ secretion in the presence of $EL4hCD19^+$ tumor cells ($p = 0.0009$) (Figure 5B). These endogenous T cells were negatively impacted in the presence of blocking MHC-I antibodies but not MHC-II blocking antibodies (Figure 5C), suggesting that broadening of the anti-tumor response was T cell receptor (TCR)/MHC-I restricted. To corroborate these results, we conducted similar experiments utilizing CAR^- splenocytes derived from mice treated with Thy1.1-derived CAR T cells injected into $EL4hCD19^+$ tumor-bearing syngeneic mice. Thy1.1-negative splenocytes were FACS sorted (Figure S6B), cocultured *in vitro* with either $EL4hCD19^+$ or $EL4hCD19^-$ tumor cells, and assayed for IFN-γ cytokine production. CAR^- splenocytes derived from mice treated with 19m28mz-mIL18 CAR-treated mice secreted increased

amounts of IFN-γ in both EL4hCD19^{+} (Figure 5D) and EL4hCD19^{-} (Figure 5E) coculture experiments ($p = 0.005$ and $p = 0.009$, respectively). Taken together, these experiments demonstrate that 19m28mz-mIL18 CAR T cells are capable of recruiting endogenous anti-tumor immune effector cells and broadening the anti-tumor response beyond the CAR target.

Next, to investigate the role of other endogenous immune cells and their potential contribution to long-term survival after IL-18 CAR therapy, we depleted either host macrophages or NK cells. Depletion of murine macrophages (Figure S7A) prior to inoculation with EL4hCD19^{+} tumor cells followed by treatment with 19m28mz-mIL18 CAR T cells led to a significant decrease in long-term survival ($p = 0.03$) (Figure 5F). Clodronate alone did impact survival in tumor-bearing mice (Figure S7B). Interestingly, depletion of NK cells (Figure S7C) prior to 19m28mz-mIL18 CAR T cell inoculation did not interfere with long-term survival (Figure 5G). These results demonstrate that despite the fact that 19m28mz-mIL18 CAR T cells activate both macrophages and NK cells in the bone marrow, only macrophages display significant anti-tumor activity and act in combination with the CAR T cells to optimally eradicate tumor cells *in vivo.*

DISCUSSION

In this study, we demonstrate that CAR T cells further modified, or armored, to secrete the immune-modulatory IL-18 cytokine exhibit enhanced expansion, persistence, and anti-tumor cytotoxicity compared to T cells modified to express the tumor-targeted CAR alone. Furthermore, these armored CAR T cells, through targeted delivery of IL-18 to the tumor, modulate the EL4 tumor microenvironment, recruit, and activate endogenous anti-tumor immune effector cells, which in turn orchestrate an effective and comprehensive anti-tumor endogenous immune response, including broadening the anti-tumor response beyond the CAR target.

Based on current clinical trial studies in patients with B cell malignancies treated with CD19-targeted CAR T cells, limitations with respect to magnitude and durability of responses seem to correlate with *in vivo* CAR T cell expansion and persistence. Furthermore, relapses after initial responses correlate with CAR-targeted antigen escape variants. In the solid tumor setting, CAR T cell therapy efficacy is likely further limited by a highly immunosuppressive tumor microenvironment scaffolding the tumor (Brown et al., 2016; Feng et al., 2017; Jackson et al., 2016; Louis et al., 2011; Wang et al., 2015). The utility of further engineering CAR T cells to secrete IL-18 is underscored by recent publications (Hu et al., 2017; Chmielewski and Abken, 2017). Hu and colleagues reported superior efficacy of IL-18-secreting CAR T cells in a xenograft model of CD19^{+} hematologic malignancies. Furthermore, they reported that this effect was mediated via autocrine stimulation of the IL-18 receptor. As the authors indicated, evaluation in immunodeficient NSG mice precluded analysis of the tumor microenvironment. In a more recent study, Chmielewski and Abken (2017) showed rejection of pancreatic cancer in a syngeneic mouse model using this approach. IL-18-enhanced CAR T cells promoted inflammatory changes in the solid tumor microenvironment by activating DCs, polarizing macrophages, and recruiting NK cells. Our studies recapitulate, bridge, and extend these findings, further enhancing our understanding of IL-18 armored CAR T cells. Consistent

with recently published data, our results demonstrate a substantial dependence on autocrine IL-18R-mediated stimulation of the CAR T cells.

Utilizing CyTOF analysis, we found that 19m28mz-mIL18 CAR T cells were not only capable of migration, and persistence in the bone marrow, but also induced endogenous CD8 T cells, macrophages, and DCs toward a more effective anti-tumor phenotype. Enhanced survival of mice inoculated with high doses of a mixed population of $hCD19^{+}$ and $hCD19^{-}$ tumor cells and treated with 19m28mz-mIL18 CAR T cells further suggest that these IL-18-secreting CAR T cells effectively recruit and activate endogenous T cells responsible for clearing $CD19^{-}$ tumor cells. We tested this hypothesis via multiple approaches and found that 19m28mz-mIL18 CAR T cells are indeed stimulating endogenous T cells to recognize both antigen-positive and antigennegative tumor cells via MHC-I/TCR engagement. Collectively, these results are suggestive of broadening of the anti-tumor response resulting from engagement of endogenous tumor-targeting immune effector cells.

Next, we demonstrate the important role of IL-18-mediated macrophage phenotype switch via pharmacological depletion of macrophages in mice treated with 19m28mz-mIL18 CAR T cells. This is consistent with literature supporting the role of pro-inflammatory M1 macrophages in suppressing tumorigenicity (Mantovani et al., 2002; Yuan et al., 2015). These results are not incompatible with data showing the importance of IL-18/IL-18R autocrine activity. Modulation of macrophages and the EL4 tumor microenvironment occurs as a secondary activity after expansion of IL-18-modified CARs. Lack of IL-18/IL-18R autocrine engagement limits this initial expansion, which leads to only modest levels of IL-18 in the TME. This explains why macrophage depletion only partially impacts efficacy, whereas IL-18 receptor deletion completely abrogates survival. In contrast, and somewhat unexpectedly, we found that despite IL-18-mediated enhanced recruitment of NK cells to the tumor site, depletion of NK cells had no apparent impact on treatment outcomes. This is not what we would have predicted based on our data and published reports by Chmielewski and Abken (2017). At this time, it is not clear whether this finding is related to the tumor model or whether this finding extends to other malignancies. This is a focus of ongoing studies.

Significantly, we have expanded our findings of IL-18 armored CAR T cells to the setting of $MUC16^{ecto}$ targeted CAR T cells in an ovarian cancertumor model. As noted, solid tumors express targeted antigens heterogeneously, making tumor escape a significant obstacle to CAR-mediated tumor eradication. Despite a known immunosuppressive tumor microenvironment in syngeneic ID8 tumor cells (Hart et al., 2011; Khan et al., 2015), we were able to show efficacy of IL-18 engineered CAR T cells in this difficult-to-treat model. The work presented here, taken together with the recently published data on IL-18 augmented CAR T cells, further expand the application of this technology to a far broader spectrum of malignancies.

EXPERIMENTAL PROCEDURES

Generation of Retroviral Constructs

SFG-19m28mz-mIL18, SFG-19mDEL, SFG-19mDEL-mIL18 CAR T cell vectors were derived from SFG-19mz constructs. SFG-1928z and 1928z-hIL18 vectors were derived from

the 19z construct, as previously described (Brentjens et al., 2003; Pegram et al., 2012). 4H11m28mz-mIL18 construct was derived from 4H11m28mz vector, as previously described (Chekmasova et al., 2010; Koneru et al., 2015). Both human and mouse IL-18 genes (Integrated DNA Technologies [IDT]) were designed and modified to include an IL-2 signal peptide and a self-cleaving 2a peptide (P2A).

T Cell Isolation and Gene Transfer

Murine T cells were isolated from splenocytes as previously described (Pegram et al., 2012). Murine CAR T cells were maintained in culture with RPMI in the presence of 100 IU/mL recombinant human IL-2 (Proleukin; Novartis, Basel, Switzerland), selected with Nylon Wool Fiber (Polysciences, Warrington, PA), and activatedwith CD3/CD28 beads, accordingto manufacturer's instructions (Gibco, Thermo Fisher, Waltham, MA). Human T cells were derived from fresh blood-derived leukocyte concentrate (Leukopack) obtained from the New York Blood Center. Mononuclear cells were separated using density gradient centrifugation with Accu-prep (axis-Shield PoC AS, Oslo, Norway). T cells were isolated, activated, and expanded with 2×10^6/mL PHA (Sigma Aldrich, St. Louis, MO). T cells were cultured in RPMI 1640 in the presence of 100 IU/mL recombinant human IL-2 (Proleukin). Viable cells were enumerated using flow cytometry and counting beads (Ebioscience), following manufacturer's protocol. Activated human and mouseT cells were retrovirallytransduced as previously described (Pegram et al., 2012, 2015). CAR expression was detected using Armenian hamster 12D11 antibody (anti-CD19 CAR) or an Alexa Fluor 647-conjugated hamster antibody that specifically binds the 4H1128z CAR (Monoclonal Antibody Facility, Memorial Sloan Kettering Cancer Center).

Proliferation, *In Vitro* Cytolysis, and Exhaustion Analysis

Proliferation assays were performed by co-culturing 1928z and 1928z-P2A-hIl-18 CAR T cells with NALM6 tumor cells at a 1:1 ratio. CAR T cell expansion was calculated on days 7 and 14, and cells were re-stimulated with NALM6 tumor cells on days 8 and 15. CAR T cell expansion was calculated using flow cytometry and counting beads (Ebioscience) according to manufacturer's instructions. T cell cytotoxicitywas assessed by standard ^{51}Cr release assays as previously described (Gong et al., 1999). Exhaustion analysis was performed *in vitro* by co-culturing CAR T cells with NALM6 tumor cell at 1:1 E:T ratio. CAR T cells were restimulated at days 6 and 12 with tumor cells, and analyses were performed at days 7 and 14 forthe expression of PD-1, TIM-3, and LAG-3 markers using flow cytometry.

Mice and *In Vivo* Models

Syngeneic Mice Experiments—For $CD19^+$ tumor cell experiments, syngeneic C57BL/6 $mCD19^{+/-}$ $hCD19^{+/-}$ mice aged 6–8 weeks were inoculated with 1×10^6 $EL4hCD19^+$ or 1×10^6 $EL4hCD19^+$ in combination with 1×10^6 $EL4hCD19^-$ tumor cells i.v. and treated with 2.5×10^6 or 1.2×10^6 anti-hCD19 CART cells i.v. the following day, or 2.5×10^6 on day 7. C57BL/6 $mCD19^{+/-}$ $hCD19^{+/-}$ mice were used as both donor and recipient as previously described (Pegram et al., 2012). For delayed tumor model experiments, $EL4hCD19^+$ tumor cells were inoculated on day 0 and CAR T cells were injected on day 7. Donor T cells were also isolated from IL-$18R^{-/-}$ mice (B6.129P2-Il18r1tm1Aki/J) (The Jackson Laboratory,

BarHarbor, ME). Micethatsurvived past day 150 were considered long-term survivors. Forsyngeneic ovarian mouse models, WT-C57BL/6 mice were inoculated with 1×10^7 ID8 tumor cells on day 0 and treated with 2×10^6 anti-MUC^{ecto16} CAR T cells either on day 24 or 42 after tumor inoculation. For macrophage depletion studies, transgenic mice were treated with clodronate liposome or PBS liposome as control, 300 μL intraperitoneally (i.p.) on days −2, 0, 2, and 6. For NK cell depletion studies, transgenic mice were treated with NK1.1 antibody (BioxCell, West Lebanon, NH) or anti-IgG2a (BioxCell) as control, 100 μg i.p. on days −2, 0, 2, and 6. Sample sizes were chosen based on estimates from pilot experiments and previously published results to power appropriately.

Xenograft Mice Experiments—Fox Chase CB17 (CB17.Cg-PrkdcscidLystbg-J/Crl, SCID-Beige mice) (Charles River Laboratories, Wilmington, MA) aged 6–8 weeks were i.v. inoculated with 1×10^6 NALM6-GFP$^+$/Luc$^+$ tumor cells on day 0 and on day 1, treated with one systemic infusion of 5×10^6 CAR$^+$ human blood-derived T cells. Bioluminescent imaging was achieved using the Caliper IVIS imaging system and analyzed with Living Image 4.0 software (PerkinElmer, Waltham, MA). Tumor-bearing mice were injected intraperitoneally with D-Luciferin (Goldbio Technology, St. Louis, MO) (150 mg/kg) and after 10 min were imaged under isoflurane anesthesia. Image acquisition was achieved using a 25-cm field of view, medium binning level, and 60-s exposure time.

All mice were monitored for survival and were euthanized when showing signs of distress. All murine studies were done in the context of a Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee approved protocol (00-05-065).

PCR Analysis

Bone marrow samples were collected on days 35, 80, 120, and 150 from mice injected with 19m28mz-mIL18 CAR T cells. Cells were lysed with TRIzol (Life Technologies, Carlsbad, CA), followed by RNA extraction using extraction kit (Life Technologies). RNA samples were converted to cDNA using first-strand cDNA synthesis kit (New England Biolabs, Ipswich, MA) and proceeded to PCR analysis using an Eppendorf cycler (Eppendorf, Hauppauge, NY) and primers specific to anti-CD19 CAR T cells (SFGFwd, agaacctagaacctcgctggaaag, and mZetaRev, gtgcattgtatacgccttcctgggggt). Estimated final PCR product size was 1,450 bp.

Mass Cytometry (CyTOF)

Mass Cytometry Antibodies—Asummary of all mass cytometry antibodies, reporter isotopes, and concentrations used for analysis can be found in Table S1. Primary conjugates of mass cytometry antibodies were prepared using the MaxPAR antibody conjugation kit (Fluidigm) according to the manufacturer's recommended protocol. Mass-tag cellular barcoding was performed as previously described (Zunder et al., 2015). Briefly, 1×10^6 cells from each animal were barcoded with distinct combinations of stable Pd isotopes chelated by isothiocyanobenzyl-EDTA in 0.02% saponin in PBS. Samples from any given tissue from one mouse per treatment group were barcoded together, with at least three biological replicates per treatment group across different plates.

Scaffold Map Generation

Total live leukocytes (excluding erythrocytes) were used for all analyses. Scaffold maps were then generated as previously reported (Spitzer et al., 2015). Briefly, we chose the bone marrow data from mice receiving wildtype CAR T cells to spatialize the initial Scaffold map. A graph was constructed by first connecting together the nodes representing the manually gated landmark populations and then connecting to them the nodes representing the cell clusters as well as connecting the clusters to one another. Each node is associated with a vector containing the median marker values of the cells in the cluster (unsupervised nodes) or gated populations (landmark nodes). Edge weights were defined as the cosine similarity between these vectors after comparing the results from the implementation of several distance metrics. Edges of low weight were filtered out. We experimented with different threshold values for the weights, and we found values of 0.8 for the initial subgraph of landmark nodes, and 0.7 for the complete graph to produce satisfying results.

IFN-γ ELISPOT Assay

The Mouse IFN-gamma ELISPOT set (Invitrogen) was used for an IFN-γ ELISPOT assay. C57BL/6 syngeneic mice splenocytes were transduced with various CAR constructs and injected on day 1 i.v. into EL4 tumorbearing mice. On day 7, splenocytes were collected and sorted for $CD3^+$ CAR^- population. 1×10^5 splenocytes were cocultured with 1×10^5 $EL4hCD19^+$ and EL4hCD19- tumor cells. For MHC blocking experiments, MHC-I (Thermo Fisher Scientific, catalog #MA1-81644) or MHC-II (Thermo Fisher Scientific, catalog #16-5321-81) blocking antibodies were added to the T cell/tumor coculture at 10 ng/mL concentrations for the duration of the experiment. The experiments were conducted according to manufacturer's instructions. The number of spots was evaluated in blinded fashion by ZellNet Consulting (Fort Lee, NJ) with a KS ELISPOT Reader System (Thornwood, NY), Software Version KS ELISPOT 4.9. Parameters were adjusted following the International Harmonization Guidelines for ELISPOT plate evaluation.

NanoString Technology Gene Expression Analysis

CAR T cells were co-cultured with tumor cells at a 1:1 ratio and flow sorted after co-culturing for 1 day or 8 days with a second tumor stimulation on day 7 of culture. Total RNA was extracted with Ambion RNA extraction kit (Life Technologies) according to manufacturer's instructions. RNA expression levels of 770 genes was detected using nanoString array (nCounter, Gene expression code set, PanCancer Immune Profiling Panel) and analyzed using nSolver Analysis system. Only statistically significant genes are shown ($p < 0.05$). The entire dataset has been deposited in the Gene Expression Omnibus (GEO) under accession number GEO: GSE97343.

Statistical Analysis

Survival curves were analyzed using Mantel-Cox (log-rank) test and other analysis were performed using two-tailed t test (p value <0.05 considered as significant). All calculations were performed using Prism 7 (GraphPad) software. Data represent means ± SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- IL-18-secreting CAR T cells enhance anti-tumor efficacy via IL-18 autocrine stimulation
- IL-18-secreting CAR T cells favorably alter EL4 tumor microenvironment
- IL-18-secreting CAR T cells enhance the anti-tumor response of endogenous T cells
- IL-18-secreting CAR T cells are efficacious in syngeneic models without preconditioning

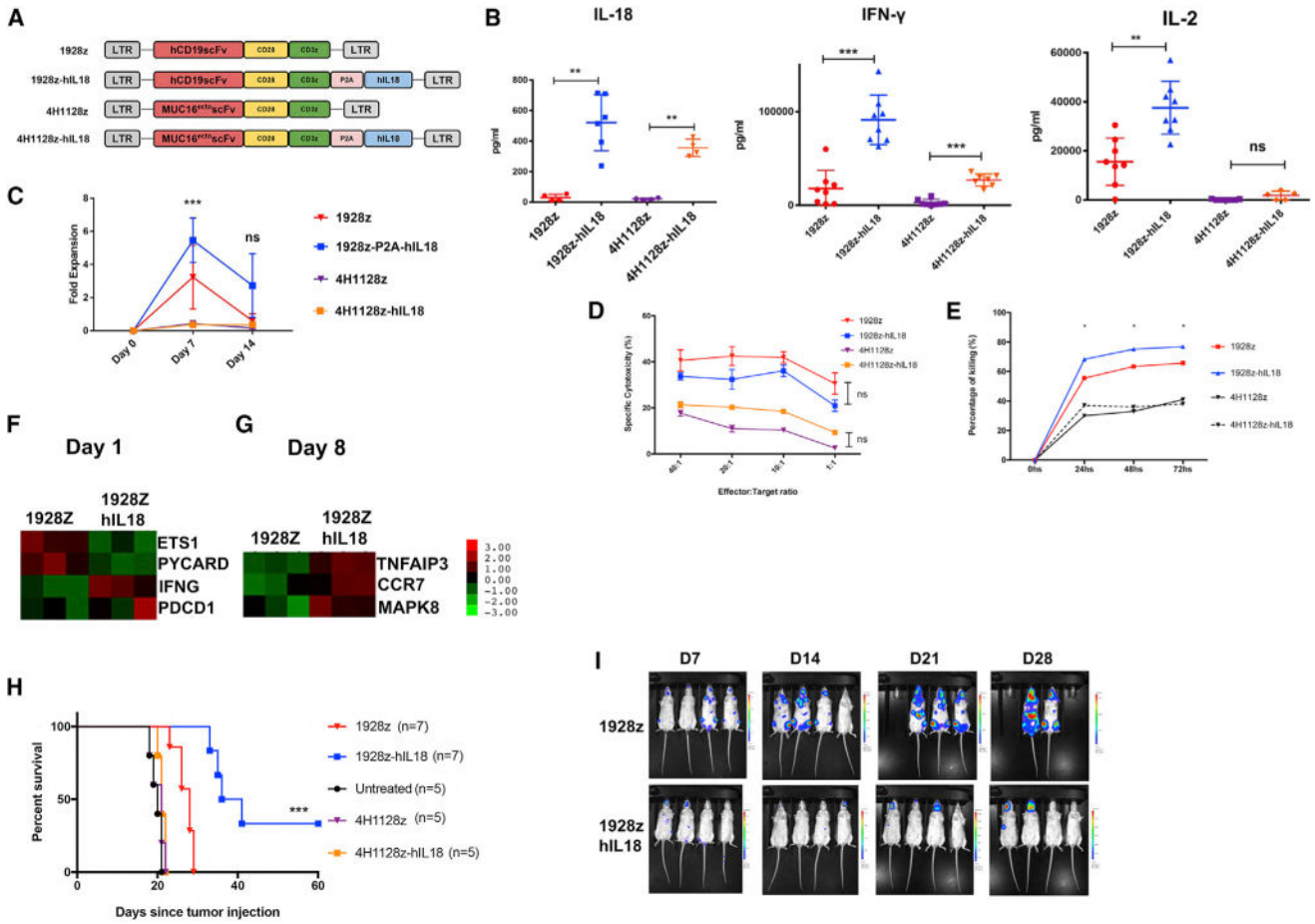

Figure 1. Human 1928z-hIL18 CAR T Cells Enhance Survival of CD19$^+$ Tumor-Bearing Scid-Beige Xenograft Mice

(A) Representation of the human CAR constructs.

(B) *In vitro* cytokine secretion of IL-18, IFN-γ, and IL-2 after coculture with NALM6 tumor cells (E:T, 1:1).

(C) *In vitro* proliferation assay with 1928z and 1928z-hIL18 CAR T cells co-cultured with NALM6 tumor cells (E:T 1:1). CAR T cells were stimulated on day 0 and day 7.

(D) *In vitro* cytotoxicity utilizing standard 4-hr ^{51}Cr release assay comparing 1928z and 1928z-hIL18 CAR T cells.

(E) *In vitro* cytotoxicity over the course of 72 hr. 1928z versus 1928z-hIL18, *$p < 0.05$.

(F and G) Statistically significant ($p < 0.05$) differential gene expression between 1928z and 1928z-hIL18 CAR T cells *in vitro* at day 1 (F) and day 8 after NALM6 tumor stimulation (G).

(H) Survival curve comparing 1928z and 1928z-hIL18 CAR T cells in a NALM6-GFP$^+$/Luc$^+$-tumor bearing xenograft Scid-Beige mouse model.

(I) Bioluminescence imaging comparing 1928z and 1928z-hIL18 CAR T cells in Scid-Beige mice inoculated with NALM6-GFP$^+$/Luc$^+$ tumor cells.

Survival curves analyses were performed using Mantel-Cox test. Other analyses were determined with Student's t test. All results were pooled from at least two independent

experiments. Data in (B)–(E) represent means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 for indicated comparison. ns, non-significant.

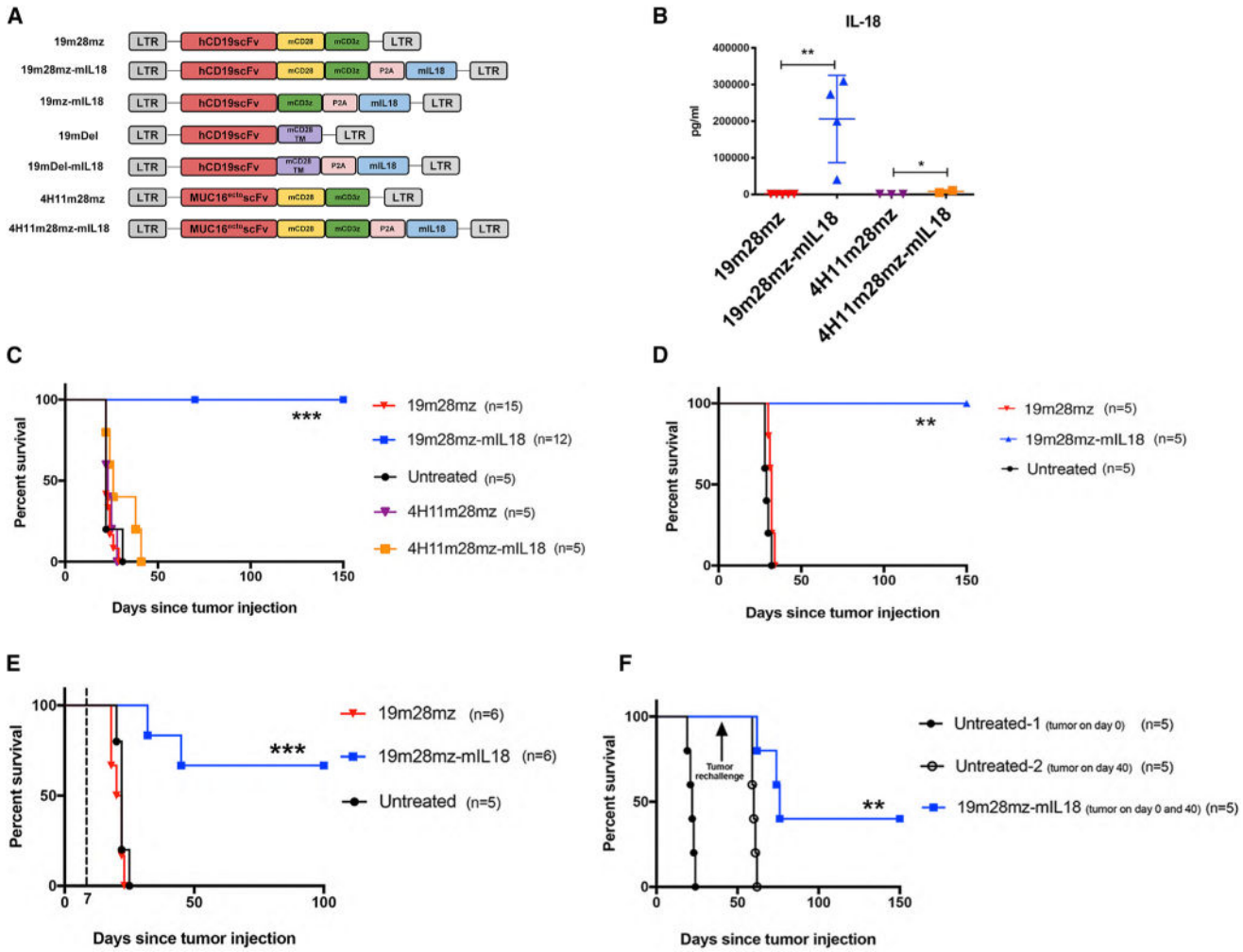

Figure 2. Murine 19m28mz-mIL18 CAR T Cells Enhance Long-Term Survival of CD19$^+$ Tumor-Bearing Syngeneic Mice

(A) Representation of murine CAR T cell constructs.

(B) *In vitro* murine IL-18 secretion after stimulation with EL4hCD19$^+$ tumor cells (E:T, 1:1).

(C and D) Survival of EL4hCD19$^+$ tumor-bearing mice treated on day 1 with (C) full dose (2.5×10^6) or (D) half dose (1.2×10^6) of CAR T cells/mouse.

(E) Survival of mice treated with 19m28mz-mIL18 CAR T cells in a delayed tumor model. Mice were inoculated with 1×10^6 EL4hCD19$^+$ tumor cells on day 0 and treated with 2.5×10^6 CAR T cells on day 7. Dashed line indicates day of CAR T cell infusion.

(F) Survival of mice inoculated with 1×10^6 EL4hCD19$^+$ tumorcellson day0, treated with 2.5×10^6 19m28mz-mIL18 CAR T cells on day 1, and then rechallenged with 1×10^6 EL4hCD19$^+$ tumor cells on day 40. Control untreated mice were inoculated with 1×10^6 EL4hCD19$^+$ tumor cells on either day 0 or day 40.

Data in (B) represent means ± SEM. Survival curves analyseswere made using Mantel-Coxtest. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for indicated comparison. All results were pooled from at least two independent experiments.

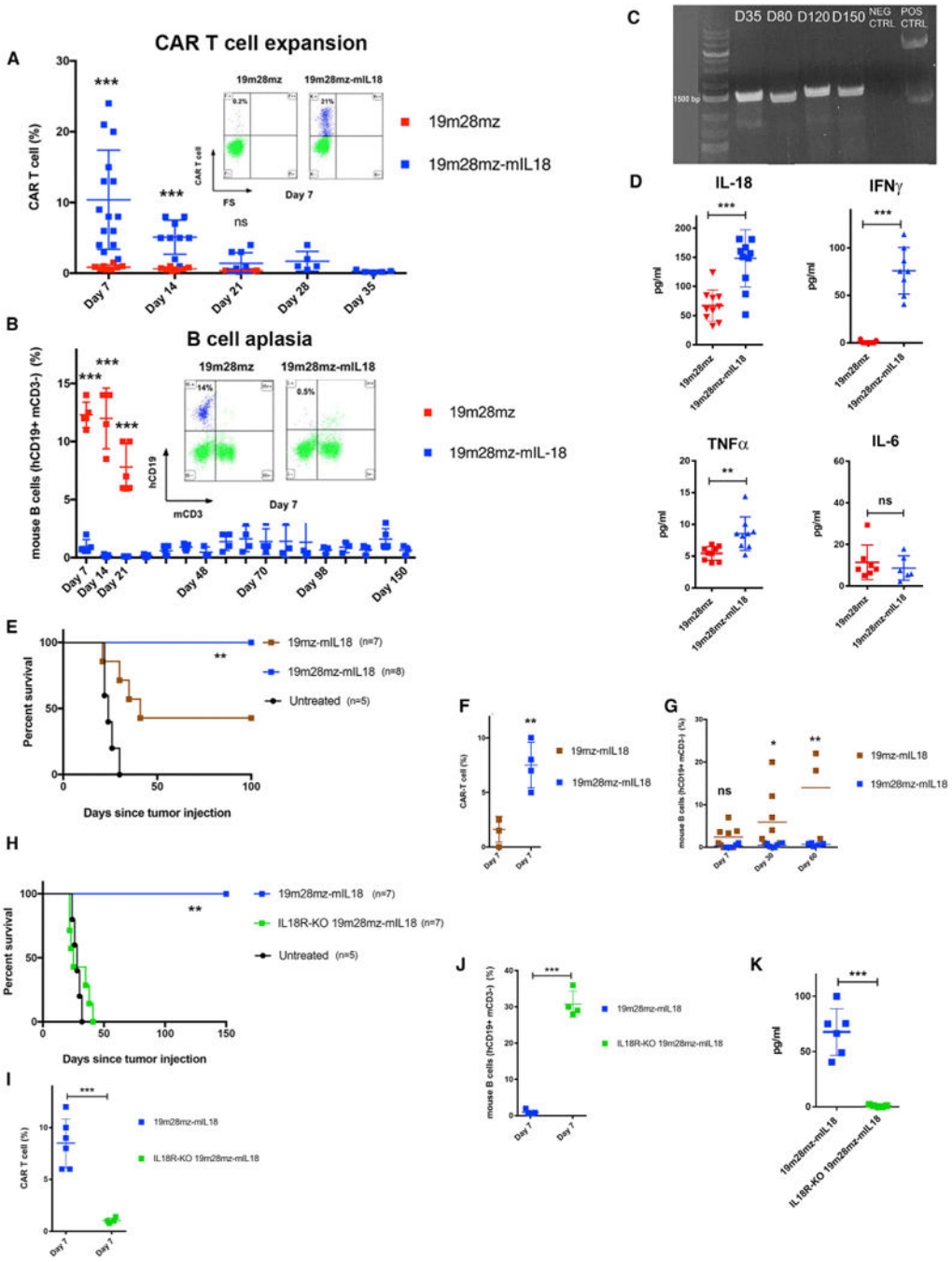

Figure 3. 19m28mz-mIL18 CAR T Cells Display Enhanced Expansion and Persistence and Depend on Autocrine IL-18 Signaling

(A) CAR T cells from peripheral blood quantified by flow cytometry.

(B) Peripheral blood B cell aplasia quantified via flow cytometry.

(C) Bone marrow PCR analysis of mice treated with 19m28mz-mIL18 CAR T cells.

(D) Serum cytokine quantification on day 7, comparing 19m28mz and 19m28mz-mIL18 CAR T cells.

(E) Survival of EL4hCD19^{+} tumor-bearing mice treated with 19mz-mIL18 or 19m28mz-mIL18.

(F) Quantification of day 7 peripheral blood CAR T cell *in vivo.*

(G) Day 7, 30, and 60 peripheral blood B cell aplasia.

(H) Survival of EL4hCD19^{+} tumor-bearing mice treated with IL18R-KO 19m28mz-mIL18 or WT 19m28mz-mIL18 CAR T cells.

(I) Day 7 peripheral blood CAR T cell quantification.

(J) Day 7 peripheral blood B cell aplasia.

(K) Day 7 serum IFN-γ quantification.

Survival curves comparisons were made using Mantel-Cox test. All results were pooled from at least two independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for indicated comparison. ns, non-significant.

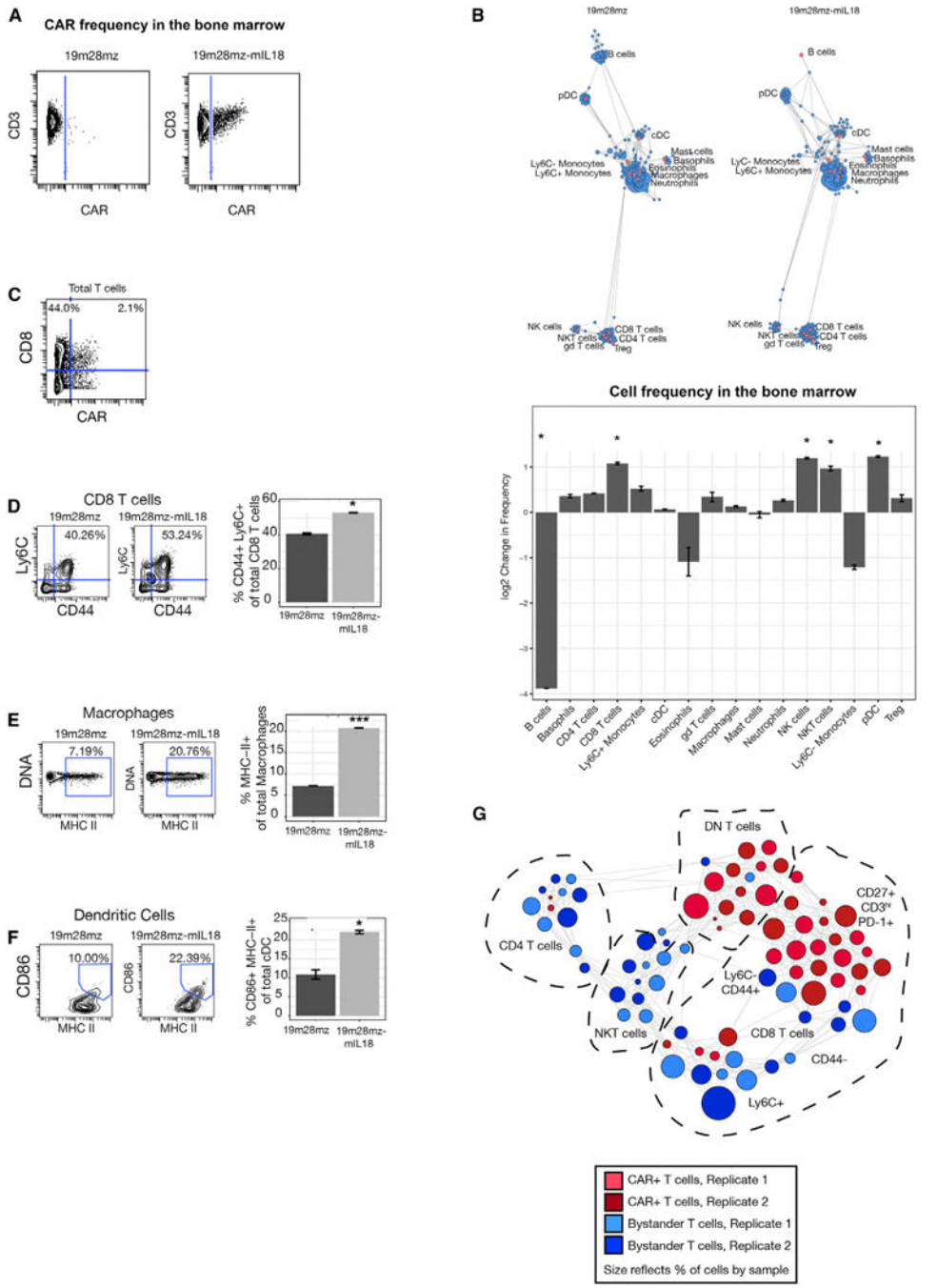

Figure 4. 19m28mz-mIL18 CAR T Cells Modulate the Tumor Microenvironment and Activate Endogenous Immune Cells

(A) Detection of CAR T cells in the bone marrow.

(B) Statistical scaffold map of the bone marrow. Size of unsupervised clusters denotes the relative number of cells in that grouping. Nodes in red denote landmark populations, defined manually. Blue nodes reflect unsupervised clusters of cells from raw dataset. Log_2 fold change of manually gated populations comparing bone marrow of mice treated with 19m28mz or 19m28mz-mIL18 CAR T cells with statistically significant differences noted ($p < 0.05$).

(C) Detection of endogenous CD8 non-CAR T cells and CD8 19m28mz-mIL18 CART cells in the bone marrow of mice treated with 19m28mz-mIL18 CAR T cells.
(D–F) Phenotypic analysis of bone marrow endogenous cells of mice treated with 19m28mz or 19m28mz-mIL18 CAR T cells: (D) endogenous CD8 T cells, (E) macrophages, and (F) dendritic cells.
(G) Statistical Scaffold map of the bone marrow comparing the phenotype of endogenous non-CAR T cells and 19m28mz-mIL18 CART cells in mice treated with 19m28mz-mIL18 CAR T cells.
Results are pooled data of two independent experiments. Adjustment by the Benjamini-Hochberg procedure for multiple testing. Data in (B) and (D)–(F) represent means ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for indicated comparison. ns, non-significant.

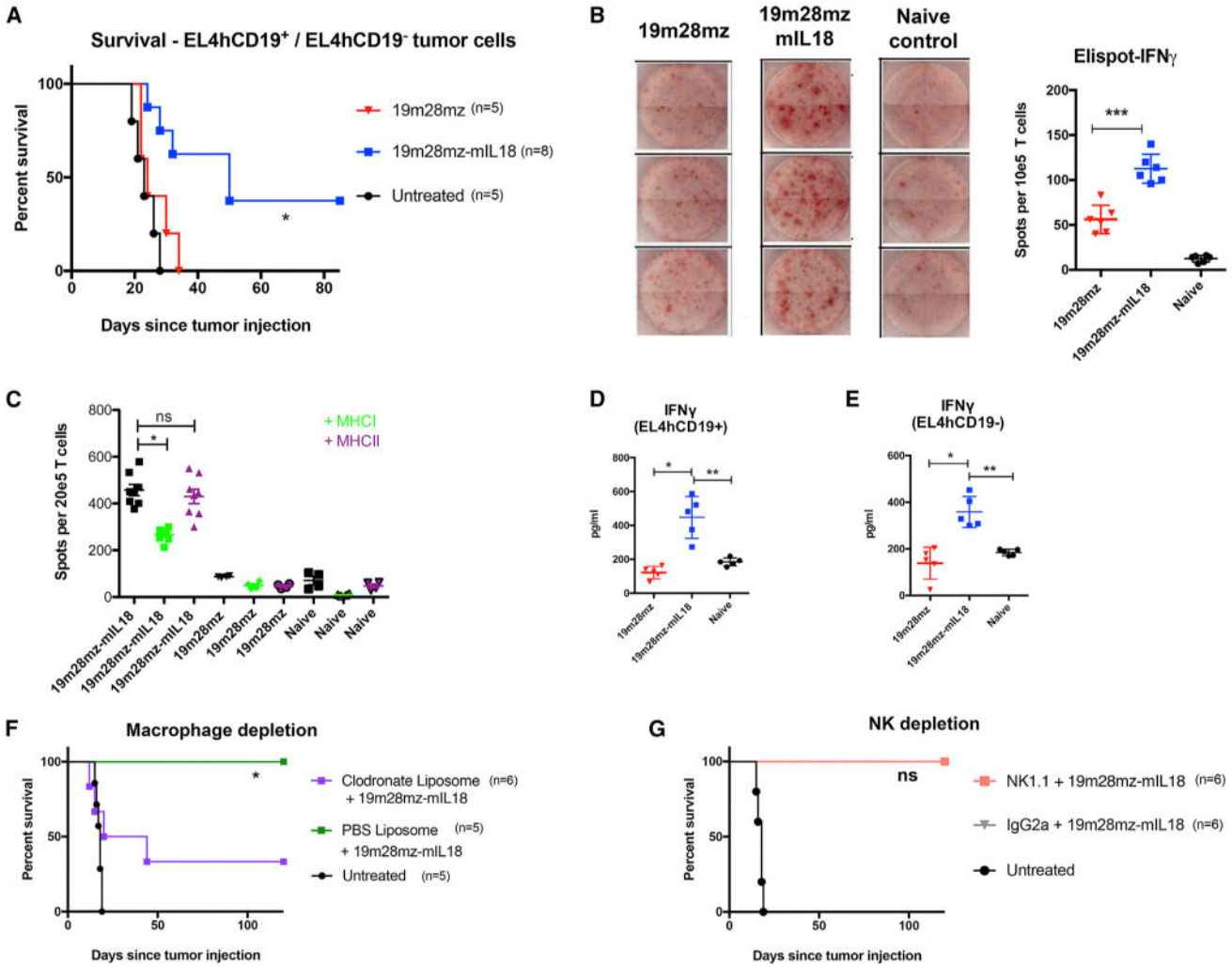

Figure 5. IL-18-Secreting CAR T Cells Engage Endogenous Anti-tumor Immune Effector Cells

(A) Survival curve of mice inoculated with both 1×10^6 EL4hCD19$^+$ and 1×10^6 EL4hCD19$^-$ tumor cells and treated on D1 with 19m28mz or 19m28mz-mIL18 CAR T cells.

(B) IFN-γ ELISPOT results comparing CD3$^+$ CAR-negative splenocytes from mice treated with 19m28mz, 19m28mz-mIL18 CAR T cells, or naive mice after coculture with EL4hCD19$^+$ tumor cells.

(C) IFN-γ ELISPOT comparing CD3$^+$ CAR-negative splenocytes from mice treated with 19m28mz, 19m28mz-mIL18 CAR T cells, or naive mice after coculture with EL4hCD19$^+$ tumor cells with/without MHC-I or MHC-II blocking antibodies.

(D) IFN-γ cytokine quantification comparing CAR-negative splenocytes after 24 hr of exposure to EL4hCD19$^+$ tumor cells (1:1 E:T ratio).

(E) IFN-γ cytokine quantification comparing CAR-negative splenocytes after 24 hr of exposure to EL4hCD19$^-$ tumor cells (1:1 E:T ratio).

(F) Survival of mice treated with 19m28mz-mIL18 CAR T cells with or without macrophage depletion.

(G) Survival of mice treated with 19m28mz-mIL18 CAR T cells with or without NK cell depletion.

Survival curves analyses performed using Mantel-Cox test. Data in (B)–(E) represent means ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for indicated comparison. ns, non-significant. All results were pooled from at least two independent experiments.